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Bifidobacterium longum as a delivery system for cancer gene therapy: Selective localization and growth in hypoxic tumors

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A fundamental obstacle in gene therapy for cancer is the specific delivery of an anticancer gene product to a solid tumor, and yet no systemic delivery system that specifically targets solid tumors currently exists. A strain of domestic bacteria, *Bifidobacterium longum*, which is nonpathogenic and anaerobic, selectively localized and proliferated in several types of mouse solid tumors after systemic application. In this report, we further describe a novel approach to cancer gene therapy in which genetically engineered *Bifidobacterium* is used as a tumor-specific vector. Similarly to wild-type *B. longum*, genetically engineered *B. longum* could be detected in tumor tissue only and was not found in a large survey of normal mouse tissues after intravenous injection. This finding strongly suggests that obligate anaerobic bacteria such as *Bifidobacterium* can be used as highly specific gene delivery vectors for cancer gene therapy. Cancer Gene Therapy (2000) 7, 269–274

Key words: Bifidobacterium longum; *anaerobic bacteria; vector; cancer gene therapy; tumor targeting; hypoxia.*

Hypoxic regions are characteristic of solid tumors in rodents[1] and occur with high frequency in many types of human tumors.[2] Tissue oxygen electrode measurements taken in cancer patients show a median range of oxygen partial pressure of 10–30 mmHg in tumors, with a significant proportion of readings below 2.5 mmHg, whereas those in normal tissues range from 24 to 66 mmHg.[3] Gene therapy in solid tumors that targets gene expression to hypoxic tumor cells is currently being investigated.[4]

It is known that certain species of anaerobic bacteria, including the genera *Clostridium* and *Bifidobacterium*, can selectively germinate and grow in the hypoxic regions of solid tumors after intravenous (i.v.) injection.[5,6] The genera *Bifidobacterium* and *Lactobacillus* are Gram-positive anaerobes and are domestic, nonpathogenic bacteria found in the lower small intestine and large intestine of humans and other animals.[7–9] These intestinal organisms have been believed to have health-promoting properties for their host, including an increase of the immune response,[10] inhibition of carcinogenesis,[11] and protection of the host against viral infection.[12] However, despite the increasing attention to these bacteria in the fields of food science, medicine, and industry, little is known about their genetic properties, mainly due to the lack of efficient and reproducible systems for genetic transfer and adequate selectable markers, especially with regard to the genus *Bifidobacterium*. Recently, an *Escherichia coli-B. longum* shuttle vector has been constructed.[13]

We propose an innovative approach to cancer gene therapy in which genetically engineered anaerobic bacteria of the genus *Bifidobacterium* are used to achieve tumor-specific gene delivery.

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MATERIALS AND METHODS

Animals

Male C57BL/6 mice (Japan SLC, Hamamatsu, Japan) of 6 to 8 weeks of age were used in this study. Mice were fed a standard rodent diet (Oriental Yeast Company, Tokyo, Japan) in the Shinshu University animal center.

Tumors

B16-F10 melanoma cells and Lewis lung cancer cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2. A total of 5×10^5 tumor cells were inoculated into the right thigh muscle of these mice. The solid tumors obtained 2 weeks after inoculation were then used for study.

Bacteria

B. longum 105-A and 108-A were anaerobically cultured at 37°C to middle log phase in slightly modified Briggs broth,[14,15] with glucose replaced by 2% lactose. The original *B. longum* was diluted 10-fold with phosphate-buffered saline (PBS) (pH 7.4); a total of 0.5 mL of the diluted suspension was then injected into the tumor-bearing mice *via* the tail vein. Immediately after injections, the suspension was quantitatively diluted and cultured as described below to determine the actual number of viable bacilli contained in the inoculum; this number was generally 5–6 × 10^6 bacilli/mouse.

Treatment of mice with lactulose

Lactulose was kindly provided by Nikken Chemicals (Tokyo, Japan) and was used as a 20% water solution after sterilization. As preliminary experiments, Lewis lung cancer tumor-bearing mice were given in a single i.v. injection of *B. longum* 9 days previously, followed by daily intraperitoneal (i.p.) administrations of 1 mL of 20% lactulose solution or 1 mL of PBS; the number of bacilli per gram of tumor tissues of the animals given lactulose was ~200-fold more than that of the mice treated with PBS (data not shown). The bacterial suspension was administered i.v. to the mice on day 0, and thereafter 1 mL of 20% lactulose was administered i.p. daily starting from day 0 to the day of sacrifice.

Preparation of tissue homogenate

At 1, 24, 48, 72, 96, and 168 hours (7 days) after injection of the *B. longum* (i.v.) and lactulose (i.p.) solutions as described above, six to eight tumor-bearing mice were sacrificed. Normal tissue samples that had been obtained from the lung, liver, spleen, kidney, and heart were used. These normal tissues and the whole tumor, which grew at the right thigh, were excised and minced thoroughly, and a sample was weighed and placed in a homogenizer to prepare a 10% homogenate with cold PBS under aseptic conditions.

Culture condition

Refrigerated, solidified culture medium (1.5% Briggs agar) was melted in boiling water, and L-cysteine and sodium ascorbate (20 mg/mL and 340 mg/mL final concentration, respectively) were added to the medium when the temperature dropped to <55°C. Thereafter, the medium was kept in a 55°C water bath ready for use. The diluted tissue homogenate, 100 μL (= 0.01 g)/dish, was inoculated into two dishes per sample and thoroughly mixed with the medium. After the agar medium was solidified at room temperature, all dishes were placed in a completely airtight desiccator at 37°C under anaerobic conditions. On day 3 of culture, the number of colonies per dish was determined.

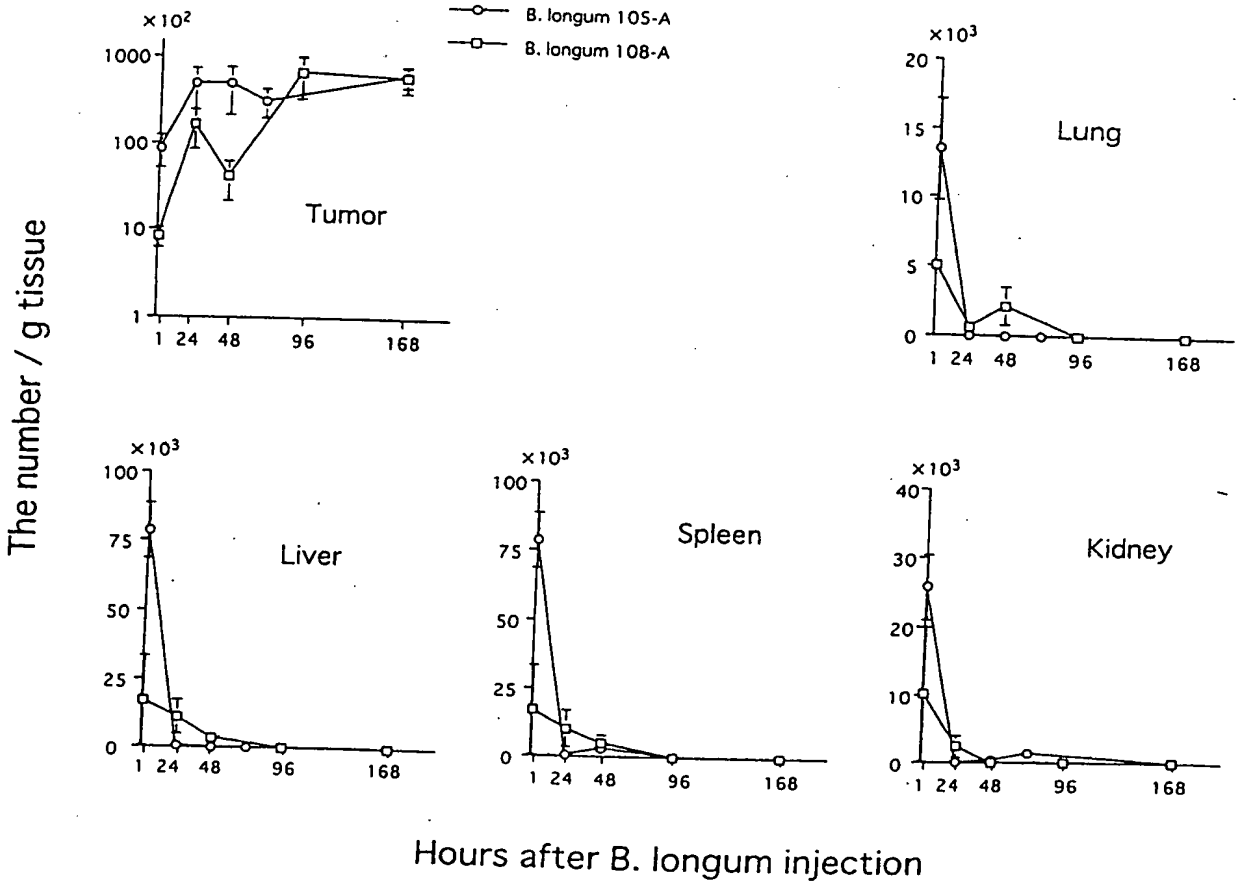

Figure 1. Organ distribution of *B. longum* 105-A and *B. longum* 108-A after a single i.v. administration of 5–6 × 10^6 viable bacilli into Lewis lung cancer-bearing mice. Each point represents the mean of the number of bacilli per gram of tissue of six to eight mice. Error bars represent 1 SEM.

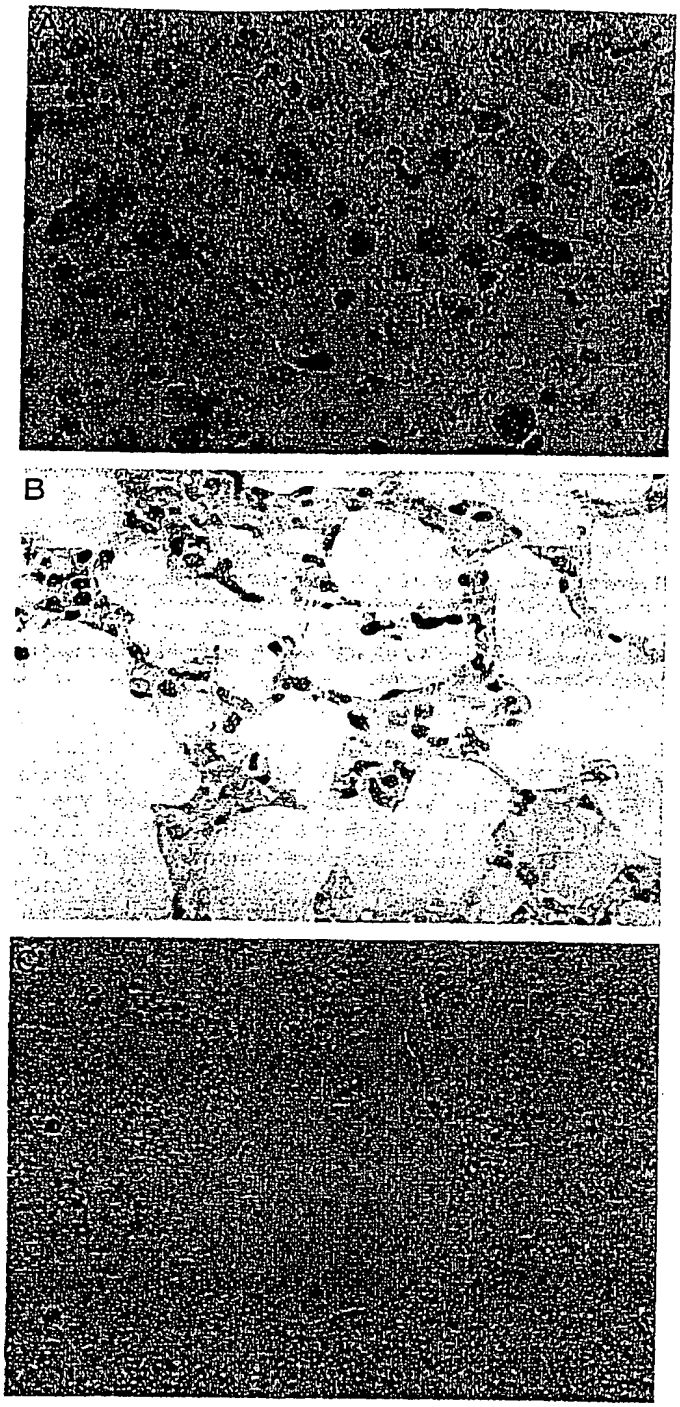

Figure 2. Photomicrograph of Lewis lung cancer tumor and normal mouse tissue sections stained by the Gram method. A: Necrotic tumor region from a mouse injected with genetically engineered *B. longum* 105-A and stained for Gram-positive rods at 168 hours postinjection. Lung (B) or liver (C) sections were also obtained from the same mouse and stained for Gram-positive rods at 168 hours postinjection, with no evidence of bacteria. All photographs are shown at ×400 magnification.

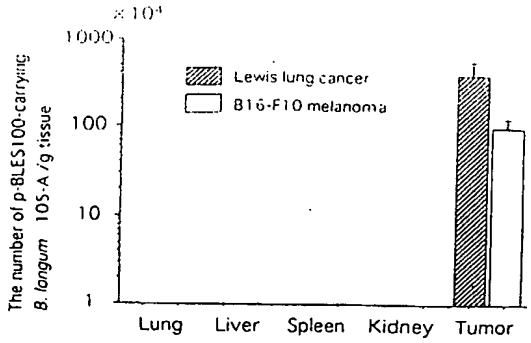

Figure 3. Number of pBLES100-carrying *B. longum* 105-A per gram of various tissues at 168 hours after a single i.v. administration of 5–6 × 10^6 viable bacilli into mice bearing Lewis lung cancer (n = 12) and B16-F10 melanoma (n = 7), which could be germinated in a medium containing spectinomycin. The mean number of bacilli is represented by a column. Error bars represent 1 SEM.

Plasmid construction and transformation of B. longum

A shuttle vector, pBLES100, was constructed by cloning a *B. longum* plasmid and a gene encoding spectinomycin adenyltransferase AAD(9) from *Enterococcus faecalis* into the *E. coli* vector pBR322. Full details of the plasmid construction are presented elsewhere.[13] The pBLES100 constructs were transferred directly into *B. longum* 105-A or 108-A by electroporation. Stable transformants were obtained with an efficiency of 1.6 × 10^4 and 2.6 × 10^3 transformants/μg DNA under the optimum conditions, using *B. longum* 105-A and 108-A, respectively. Transformed *B. longum* was grown under anaerobic conditions at 37°C in Briggs broth containing 75 μg/mL spectinomycin. These bacilli were injected i.v. into tumor-bearing mice as described above.

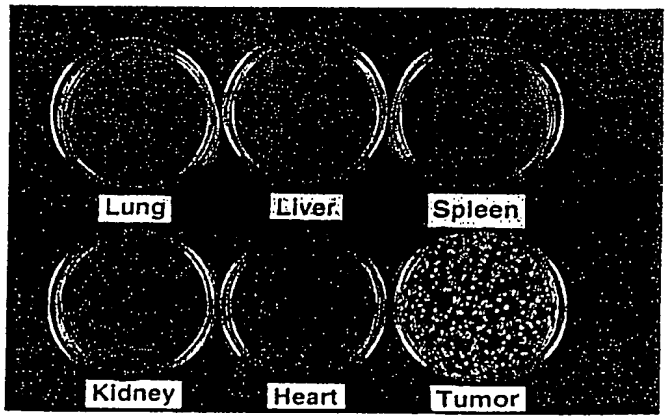

Figure 4. Comparison of the number of genetically engineered *B. longum* 105-A in both Lewis lung cancer tumors and normal tissues from mice after 168 hours. After homogenization of removed tumor and tissues, 100 μL of sample was sown in each of the spectinomycin-containing dishes and cultivated for 3 days. Approximately 400 colonies were observed in the tumor, but no colonies were present in normal tissue.

Histology

Animals were sacrificed at 168 hours after bacterium injection. Tumors and normal tissues were then excised, fixed in 10% formalin solution, sectioned in paraffin, and stained with a Gram's stain.

Spectinomycin-resistant gene delivery into the hypoxic tumors

The mice bearing a B16-F10 melanoma with administration of transformed *B. longum* 105-A with spectinomycin-resistant gene were divided into two groups: (a) four mice to which spectinomycin (200 mg/kg) was administered i.p. daily starting from day 1 to day 3 and sacrificed on day 4; (b) as a control, four mice were given PBS instead of spectinomycin. The mice with administration of wild-type (wt) *B. longum* 105-A were also divided; four mice each were placed in the spectinomycin and control groups. Tumors of each group were excised and cultured without the spectinomycin conditions. For statistical analysis, the Mann-Whitney *U* test (Statview-J4.11, Abacus Concepts, Berkeley, Calif) was used to compare the number of bacilli between treatment groups receiving spectinomycin or PBS. A P value of $<.01$ was considered significant.

RESULTS

Selective growth of unmodified B. longum *in tumor tissues*

Figure 1 shows the number of *B. longum* 105-A and 108-A organisms per gram of various tissues at various time intervals after i.v. administration of $5–6 \times 10^6$ viable bacilli into mice bearing Lewis lung cancer tumors. At 168 hours, tumors had ~60,000 bacilli per gram of tumor tissue regardless of the bacterial strain used. In contrast, the number of *B. longum* organisms in nonmalignant tissues, such as the liver, spleen, kidney, and lung from the tumor-bearing mice, began to decrease immediately after injection. Bacilli were below detectable levels in all normal tissues after 168 hours with *B. longum* 105-A and after 96 hours with *B. longum* 108-A. We examined the number of *B. longum* 108-A per gram of various tissues at 96 hours after i.v. administration of $5–6 \times 10^6$ viable bacilli into B16-F10 melanoma tumor-bearing mice. Bacilli were detected in the tumor tissue only but not in normal livers, spleens, kidneys, or lungs (data not shown).

Histology

Lewis lung cancer tumor-bearing mice were injected i.v. with wt *B. longum*, killed 168 hours later, and examined for the presence of Gram-positive bifidobacterial rods in both tumors and normal tissues. Numerous bacilli were scattered in the necrotic region of the tumor. Gram's staining of histological sections indicated that *B. longum* did not germinate in normal tissues, including the lung, liver, spleen, kidney, and heart (Fig 2, A–C).

Selective growth of genetically engineered B. longum *in tumor tissues*

The wt *B. longum* strain 105-A was transfected with plasmid pBLES100. Both Lewis lung cancer and B16-F10 melanoma tumor-bearing mice were injected i.v. with $5–6 \times 10^6$ viable pBLES100-carrying *B. longum* 105-A, killed 168 hours later, and examined for the presence of bacilli in both tumors and several normal tissues. The number of the transformed *B. longum* 105-A per gram of various tissues of the two kinds of tumor-bearing mice are shown in Figure 3. Both Lewis lung cancer tumors and B16-F10 melanoma tumors exhibited a heavy infestation of bacilli as well as wt. However, in the normal tissues such as the lung, liver, spleen, and kidney, no bacilli were detected. As shown in Figure 4, ~400 colonies were recognized on the spectinomycin-containing agar plate; these colonies inoculated the tumor tissue only.

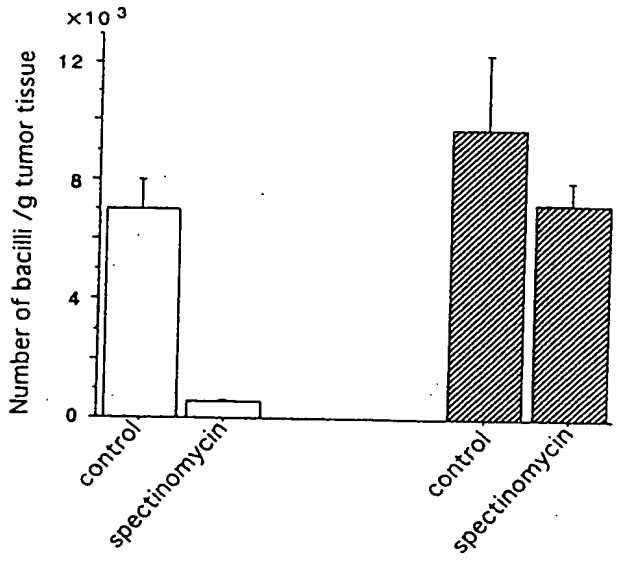

Figure 5. The number of bacilli per gram of tumor tissue at 96 hours after injection into B16-F10 melanoma-bearing mice. The mice that were administered wt *B. longum* 105-A (□) and administered transformed *B. longum* 105-A (▨) were divided into the spectinomycin and the control groups.

Spectinomycin-resistant gene delivery into the hypoxic tumors

Figure 5 shows the number of wt *B. longum* 105-A and transformed *B. longum* per gram of tumor tissue of mice bearing a B16-F10 melanoma. When spectinomycin was given to the animals injected with wt *B. longum* 105-A, the number of bacilli significantly decreased compared with the control group treated with PBS ($P < .01$). However, in mice that were injected with transformed *B. longum* carrying the spectinomycin-resistant gene, the same number of bacilli was detected compared with the PBS control group ($P = .81$).

DISCUSSION

A central problem for cancer gene therapy is the lack of specificity of current delivery systems. After i.v. inocula-

tion of *B. longum* to tumor-bearing mice, we initially observed a distribution of viable bacilli throughout the body; however, after 96–168 hours, they were detectable only in the tumor tissue. The fact that the bacilli are not only detected but can also proliferate in the tumor tissue implies that this tissue possesses an environment that is suitable for the growth of this bacterium. The only requirement for the success of this gene therapy strategy in the clinic should be the presence of hypoxia in the treated tumors. Metastasized or disseminated lesions as well as primary disease should be amenable to the treatment as long as regions of hypoxia are present. Thus, the detection of a primary tumor locus or of a metastatic focus may be diagnostically feasible with the transformation of a suitable marker gene.

This gene delivery system is not only tumor-specific but also nontoxic. Some investigators have examined the availability of anaerobic bacteria such as *Clostridia*[16–18] or *Salmonella*[19,20] as gene delivery vectors, but the pathogenicity of these organisms in humans likely precludes their use. Some reports have demonstrated febrile adverse reactions as side effects after injection with *Clostridium butyricum* spores[21] or oral intake of *Salmonella typhi*.[22,23] Conversely, *Bifidobacterium* strains are widely used for the preparation of fermented milk products in many Asiatic and Western countries. In addition to the assumed health-promoting properties of some *Bifidobacterium* species for humans, the nonpathogenicity of these microorganisms is now generally accepted. To be able to exploit the potential of these organisms for cancer gene therapy, detailed knowledge is required about such basic biological phenomena as cellular metabolism, gene expression, and protein secretion and genetics. However, studies on *Bifidobacterium* at the molecular level are severely limited in the absence of an efficient transformation system. Recently, Argnani et al[24] developed a system for the convenient and reproducible genetic transformation of strains of the genus *Bifidobacterium*. We demonstrated the tumor-specific germination of *Bifidobacteria* with transfected *B. longum* 105-A. The vector pBLES100 has two unique restriction sites, *Eco*RI and *Hin*dIII, which are suitable for gene cloning. These results strongly suggest that *B. longum* can be used as a highly specific gene delivery vector for cancer therapy. In the present study, spectinomycin-resistant gene, which is carried in pBLES100, functions *in vivo*, as shown in Figure 5; this finding indicates that any genes cloned in pBLES100, which is advantageous in the treatment of cancer, could be translated in *B. longum* specifically delivered to tumor tissues.

As a further safeguard for this gene delivery system, *Bifidobacterium* can be killed easily by antibiotics. We confirmed that both wt and genetically engineered *B. longum* were killed with 50 μg/mL ampicillin *in vitro* (data not shown).

In summary, we have demonstrated that the genus *Bifidobacterium*, which is beneficial rather than nonpathogenic for its host, can be engineered. When these genetically engineered *Bifidobacteria* were introduced systemically into tumor-bearing mice, bacteria were found only in the tumors, presumably due to the hypoxic environment required for the growth of these bacteria. We believe that this novel approach for tumor targeting using *Bifidobacteria* could be useful for gene therapy of solid tumors.

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